

Joel Roy, France. Oil on canvas.

## ANNEX 1

# Angiogenesis and Cancer Control: From Concept to Therapeutic Trial

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*Current research into angiosuppressive agents is providing cautious optimism in the field of clinical cancer research.*

**Background:** There is extraordinary interest in developing angiosuppressive agents for cancer treatment. Several new agents appear promising for the treatment of a variety of human cancers. Current concepts and new agents in clinical trials are the focus of this article. In particular, the introduction of a new treatment for human brain tumors is presented in detail, using an antiangiogenic agent, penicillamine, and depletion of an obligatory cofactor of angiogenesis, copper.

**Methods:** The explosive increase in literature on antiangiogenesis is reviewed using computerized search, findings presented at the recent national cancer and angiogenesis meetings. A specific protocol, NABTT 97-04, "Penicillamine and Copper Reduction for Newly Diagnosed Glioblastoma," is presented as an example of angiotherapeutic drug discovery.

**Results:** A number of promising molecular approaches are being introduced to suppress tumor angiogenesis. Major categories of angiogenesis antagonists include protease inhibitors, direct inhibitors of endothelial cell proliferation and migration, suppression of angiogenic growth factors, inhibition of endothelial-specific integrin/survival signaling, chelators of copper, and inhibitors with specific other mechanisms. The preliminary results of early trials offer a glimpse into how antiangiogenesis therapy will be integrated into future care of the patient with cancer.

**Conclusions:** Thirty-five antiangiogenesis therapies are currently being evaluated in clinical trials. As we learn more about the fundamental mechanisms of angiogenesis, eg, the role of copper in growth factor activation, effective methods of cancer control will be implemented.

## Introduction

One of the great scientific questions of this century remains: What controls the growth of blood vessels? The answer to the biological control of angiogenesis is critical to the clinical control of cancer and other angiogenesis-

dependent diseases.

Judah Folkman introduced a sweeping hypothesis in 1971.<sup>1</sup> A quarter century later, the paradigm has evolved (Table 1) to accommodate the stream of discoveries coming from not only the Harvard laboratory, but also the National Cancer Institute (NCI) and research centers worldwide. In recent years, with the pace of discovery increasing, the search for an effective, nontoxic, antiangiogenesis therapy appears to be within the grasp of oncology. Antiangiogenic therapy is a high priority for the NCI, especially in the wake of the description of angiostatin and endostatin<sup>2-5</sup>

Research in antiangiogenesis therapy currently is robust with numerous promising avenues. More time, however, is required to be able to identify the appropriate inhibitor, to meld angiosuppression with the current therapeutic modalities, and to design optimal formulations, routes of administration, and dosing schedules. The results of the current clinical trials<sup>6</sup> will shape Food and Drug Administration approval of antiangiogenesis therapy in the near future. Certain drugs, eg, thalidomide, are being used clinically on a limited, "off-label" basis.

## Evolution of the Paradigm of Tumor Angiogenesis

The major changes in the paradigm of tumor angiogenesis are summarized in Table 1.

Table 1. — Evolution of the Paradigm of Tumor Angiogenesis		
	Original (1971)	Current (1999)
Angiogenesis Dependency	Tumor growth is angiogenesis-dependent; each increment of tumor growth requires an increment of capillary growth.	Concept validated in hundreds of experiments, using genetic, pharmacological, and physiological control mechanisms.  Concept extended to other "angiogenesis diseases," eg, diabetic retinopathy, atherosclerosis, rheumatoid arthritis, and psoriasis.
Cell-Cell Signaling	Tumor cell stimulates endothelial cell Proliferation	Tumor cell stimulates endothelial cell proliferation. Also, there is paracrine, reciprocal, bi-directional cellular activation.  Endothelial cell stimulates tumor cell growth.
Molecular Mediator ("Factor") of Angiogenesis	"Tumor angiogenesis factor" produced by tumor cell.	"Dormancy therapy" validated in numerous animal models.  Regression inhibitors observed when combination of angiogenesis inhibitors is used or when antiangiogenesis is used in combination with classic cytotoxic/radiation therapy.
Switch to Malignant Phenotype	Switch to malignancy is characterized by onset of angiogenesis.	Switch to malignant phenotype is linked to angiogenesis.  Numerous signaling systems are involved in oncogenesis, and many of these involve angiogenesis stimulators.
Control of Angiogenesis	Predominantly under the control of an angiogenesis factor produced by tumor.	Tightly coordinated "balancing act." Numerous oncogenes/suppressor genes, growth factors, and endogenous inhibitors, proteases and protease inhibitors, and trace elements that can switch angiogenesis "on" or "off."
Control of Solid Tumor Growth	Solid tumors larger than 2 mm require blood supply for further growth.	Antiangiogenesis therapy limits solid tumor growth but also may be valuable for control of leukemia and myeloma.
Role of Apoptosis	Angiogenic inhibitors block endothelial cell proliferation.	Angiogenic inhibitors can inhibit endothelial cell proliferation, but also can induce apoptosis in the endothelial cell population and/or increase apoptosis in the neighboring tumor cell population, even if microvascular density is unchanged.
Angiogenesis and Invasion	Invasion and metastatic spread are angiogenesis-dependent.	Angiogenesis and tumor cell invasion/metastatic spread are closely linked. Both require proteolytic degradation of extracellular matrix.  Angiogenesis antagonists suppress invasiveness and metastatic spread.

## Angiogenesis Dependency of Tumor Growth

The central concept that tumor growth is "angiogenesis dependent"<sup>7</sup> is well accepted today, with more than 2,500 scientific reports showing angiogenesis linked to tumor growth.<sup>8</sup> Stated concisely, "every increment of tumor growth requires an increment of vascular growth."<sup>9</sup>

### Tumor Angiogenesis Factors

Before the term "cell-cell signaling" became fashionable, Folkman postulated the existence of a specific protein that tumor cells secrete to stimulate capillary endothelial cell proliferation. Such a molecule, if identified, could be a target for therapy. Numerous angiogenic genes and gene products, from both neoplastic and normal tissues, have been isolated, purified, cloned, and produced using recombinant DNA technology (Table 2).

Table 2. — Endogenous Stimulators and Inhibitors of Angiogenesis		
	Stimulators	Inhibitors
Growth Factors	Angiogenin Angiotropin Epidermal growth factor Fibroblast growth factor (acidic and basic) Granulocyte colony-stimulating factor Hepatocyte growth factor/scatter factor Platelet-derived growth factor-BB Tumor necrosis factor-alpha Vascular endothelial growth factor	
Proteases and Protease Inhibitors	Cathepsin Gelatinase A, B Stromelysin Urokinase-type plasminogen activator (uPA)	Tissue inhibitor of metalloprotease (TIMP-1, TIMP-2)  Plasminogen activator-inhibitor-1 (PAI-1)
Trace Elements	Copper	Zinc
Oncogenes	c-myc ras c-src v-raf c-jun	p53 Rb
Signal Transduction Enzymes	Thymidine phosphorylase Farnesyl transferase Geranylgeranyl transferase	
Cytokines	Interleukin-1 Interleukin-6 Interleukin-8	Interleukin-10 Interleukin-12
Endogenous Modulators	Alpha v Beta 3 integrin Angiopoietin-1 Angiostatin II (AT1 receptor) Endothelin (ETB receptor) Erythropoietin Hypoxia Nitric oxide synthase Platelet-activating factor Prostaglandin E Thrombopoietin	Angiopoietin-2 Angiotensin Angiotensin II (AT2 receptor) Caveolin-1, caveolin-2 Endostatin Interferon-alpha Isoflavones Platelet factor-4 Prolactin (16 Kd fragment) Thrombospondin Troponin-1

It is now recognized that the endothelial cell, by paracrine mechanisms, produces growth factors that stimulate the proliferation of the tumor cell population. Thus, there is a bi-directional reciprocal signaling of endothelial and tumor cell growth. The major targets of pharmacologic therapies are vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). Overall, angiogenesis can be viewed as the result of a complex "yin-and-yang" balance of tightly regulated oncogenes and suppressor genes, stimulatory and inhibitory peptides, proteases and endogenous inhibitors, and microenvironmental factors such as the level of oxygen or copper ion.

## *Dormancy Therapy*

Experiments where tumors were transplanted into an avascular environment proved that angiogenesis was a control point in tumor growth. Tumors deprived of angiogenesis remained dormant indefinitely; rapid logarithmic growth followed the acquisition of a blood supply.<sup>10</sup> "Dormancy therapy" remains a powerful concept<sup>4,11-13</sup>

## *Solid Tumors and Hematopoietic Tumors*

Recent data suggest that antiangiogenesis not only will be useful in the control of solid tumors,<sup>14</sup> but also may be valuable as therapy for patients with leukemia and myeloma.<sup>15-17</sup>

## *Switch to the Malignant Phenotype*

In numerous models, the original concept that the switch from premalignant to malignant lesions is linked to angiogenesis<sup>18,19</sup> is validated with elegant models demonstrating a cohort of specific angiogenic genes, growth factors, peptides, and proteases activated during malignant transformation.<sup>20-22</sup>

## *Role of Apoptosis*

Angiogenic inhibitors suppress endothelial cell proliferation,<sup>23,24</sup> but another important control mechanism is the induction of endothelial cell apoptosis. Furthermore, even without changes in microvascular density, apoptosis of tumor cells occurs in proximity to endothelium following treatment with angiogenesis inhibitors.<sup>22</sup>

## *Angiogenesis and Invasiveness*

Initially, invasion and metastatic spread were viewed as angiogenesis-dependent events.<sup>1,14</sup> Reinforcing this concept is the increasing attention placed on the role of matrix proteases that control endothelial cell migration and tubule formation as well as tumor cell migration and spread. From a practical perspective of drug discovery and therapy, most angiogenesis inhibitors also act as anti-invasive or antimetastatic compounds.<sup>25,26</sup>

## *Deciphering the Angiogenesis Code*

The list of known angiogenic stimulators and inhibitors grows yearly.<sup>27</sup> Listed in Table 2 are 35 activators and 18 angiosuppressants. The activators can be divided into seven categories: (1) growth factors, (2) proteases, (3) trace elements, (4) oncogenes, (5) cytokines, (6) signal transduction molecules, and (7) endogenous inducers.

The principal growth factors driving angiogenesis are VEGF, bFGF, and hepatocyte growth factor/scatter factor.<sup>28-30</sup> Other positive regulators are angiopoietin-1, angiotropin, angiogenin, epidermal growth factor, granulocyte colony-stimulating factor, interleukin-1 (IL-1), IL-6, IL-8, platelet-derived growth factor (PDGF), and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ).<sup>27,31</sup> Matrix proteins such as collagen and the integrins are critical to angiogenesis. Several proteolytic enzymes critical to angiogenesis and tumor spread include cathepsin, urokinase-type plasminogen activator, gelatinases A/B, and stromelysin.<sup>26,32</sup>

Angiogenesis is physiologically suppressed by one or more of the known endogenous inhibitors, including angiopoietin-2, angiostatin, endostatin, interferon- $\alpha$ , kringle-5, platelet factor-4, prolactin (16kD fragment), thrombospondin, tissue inhibitors of metalloproteinase (TIMP-1, TIMP-2 and TIMP-3), and troponin I.<sup>27,33,34</sup>

TNF- $\alpha$ , transforming growth factor- $\beta$  (TGF- $\beta$ ), or IL-4 are bifunctional modulators. These molecules are either stimulators or inhibitors depending on the amount, the site, the microenvironment, the presence of other cytokines, etc.

Recent attention has been focused on the role of the p53 tumor-suppressor gene in angiogenesis. The p53 gene is inactivated in over 50% of all human cancers. Mutant p53 correlates with reduced expression of

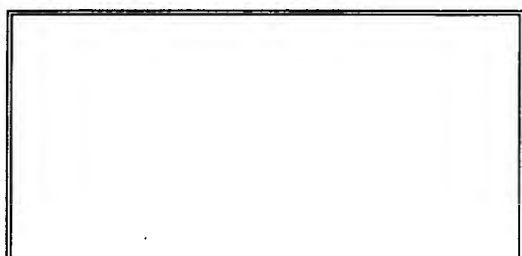
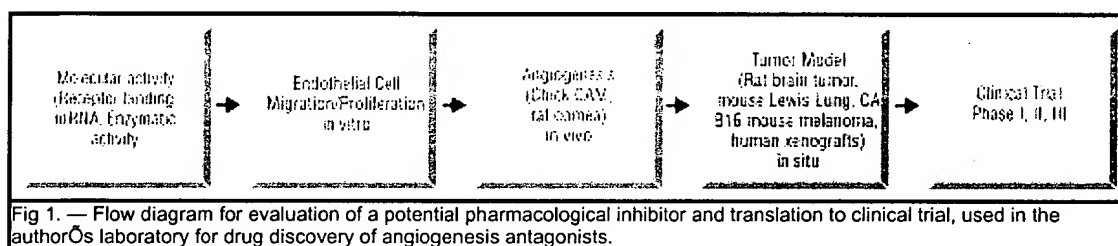
thrombospondin-1, increased angiogenesis, and malignant progression.<sup>35</sup> Exogenous expression of wt-p53 inhibits angiogenesis in vivo resulting in the formation of dormant tumors.<sup>13</sup> By inhibiting angiogenesis, p53 indirectly induces apoptosis in vivo but not in vitro and can revert tumors to a dormant phenotype.<sup>13</sup>

One of the earliest genetic alterations, for example, in human astrocytoma progression is mutation of the p53 tumor suppressor gene; transfection of wild-type p53 into glioblastoma cells leads to angiosuppression. The transfected cells secrete a factor that neutralizes bFGF and the angiogenicity of the parent glioblastoma cells.<sup>36</sup> As nonmalignant fibroblasts progress to tumorigenicity, cells become fully angiogenic in two steps. First, there is loss of both alleles of wild-type p53, which causes a 20-fold drop in secreted thrombospondin and a fourfold increase in secreted VEGF.<sup>20</sup> Second, angiogenic activity increases again on transformation by activated *ras* due to a further twofold increase in secreted protein levels and an overall angiogenic activity. Thus, there is a step-wise change in the angiogenic phenotype in response to oncogene activation and tumor suppressor gene loss involving a decrease in the secretion of inhibitors and the sequential up-regulation of inducers of angiogenesis.<sup>20</sup>

Other recently discovered examples of endogenous inhibitors include caveolin-1 and -2. These molecules are highly expressed in endothelial cells. Angiogenic growth factors (bFGF, HGF/SF, VEGF) each down-regulate the expression of caveolin. Angiogenesis inhibitors (including angiostatin, fumagillin, and thalidomide) block VEGF-induced caveolin expression.<sup>37</sup>

## Angiogenesis Drug Discovery

The basic algorithm we use for translating a lead compound from the laboratory to the clinic requires five steps (Fig 1). (1) First, there is a defined molecular target, eg, a growth factor receptor, or interference with signal transduction, or inhibition of a critical enzyme. The putative inhibitor would be tested for functional activity, eg, receptor binding, or inactivation of mRNA, or neutralization of enzymatic activity. Such assays could also be used for rapid throughput for screening of potential compounds. For example, Gross et al<sup>38</sup> hypothesized that an angiogenic inhibitor, to be effective, would likely inhibit the plasminogen-plasmin system, particularly urokinase (uPA) activity. This strategy of using uPA as a pharmacological target has been used successfully in our laboratory (Fig 2) to identify several inhibitors (eg, penicillamine, suramin, diaminoanthraquinone [DAAQ])<sup>39-42</sup> of clinical relevance because of the overexpression of uPA in human brain tumors.<sup>43</sup> (2) The next step is to determine in vitro if endothelial cell migration (Fig 3) and proliferation are inhibited. (3) Further studies are done with chick chorioallantoic membrane (Fig 4) that has proved to be a useful, inexpensive screening test. The "gold standard" for an angiogenic activator or inhibitor is the corneal assay. The normally avascular cornea enables quantitation of the number of vessels and the rate of vascular ingrowth. A slow-release polymer containing the inhibitor can be placed in a cornea micropocket, and it can be titrated against a known agonist such as bFGF.<sup>40</sup> (5) Finally, a rigorous test of an angiogenesis inhibitor is to evaluate it in a vascularized organ such as the brain (Fig 5).



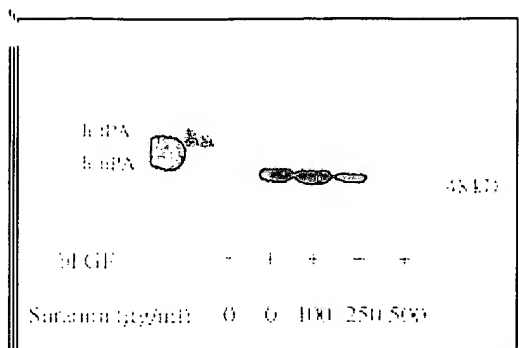


Fig 2. — Suramin inhibits protease (urokinase-type plasminogen activator) activity produced by bFGF. Protease activity can be used as a molecular screen to evaluate lead compounds. From Takano S, Gately S, Neville ME, et al. Suramin, an anticancer and angiostatic agent, inhibits endothelial cell binding of basic fibroblast growth factor, migration, proliferation, and induction of urokinase-type plasminogen activator. *Cancer Res.* 1994;54:2654-2660. Reprinted with permission.

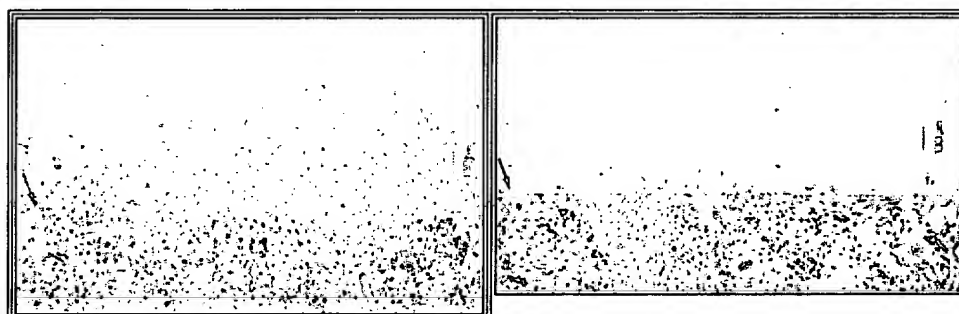


Fig 3. — Endothelial cell migration assay. In response to an angiogenesis antagonist, eg, suramin, endothelial cells fail to migrate. Left, control, without suramin. Right, shows the effect of adding the inhibitor, suramin, 500 µg/mL. The bar equals 100 µm. From Takano S, Gately S, Neville ME, et al. Suramin, an anticancer and angiostatic agent, inhibits endothelial cell binding of basic fibroblast growth factor, migration, proliferation, and induction of urokinase-type plasminogen activator. *Cancer Res.* 1994;54:2654-2660. Reprinted with permission.



Fig 4. — Chick chorioallantoic membrane assay. Note the central avascular zone where an invisible methylcellulose disc contains an angiogenesis inhibitor (suramin) causing the vessels to regress and grow away from the disc. The assay is an inexpensive in vivo screening test for an angiogenesis inhibitor.



Fig 5. — Effect of copper depletion and penicillamine on angiogenesis in a rabbit brain tumor model. Note the control (left) shows numerous coiled vessels growing into the tumor, resembling the hypervascularity of a human malignant brain tumor. By contrast (right), the treated rabbit has a normal appearing vasculature on the cerebral cortex with a pattern of delicate, orderly blood vessels. From Brem S, Zagzag D, Tsanacis AM, et al. Inhibition of angiogenesis and tumor growth in the brain. Suppression of endothelial cell turnover by penicillamine and the depletion of copper, an angiogenic cofactor. *Am J Pathol.* 1990;137:1121-1142. Reprinted with permission.

## Strategies for Therapeutic Angiosuppression

Strategies for therapeutic angiosuppression generally involve either interference with the activators of angiogenesis or amplification of the endogenous suppressors. The five classes of angiogenesis antagonists in current clinical trials include inhibitors of proteases (Table 3), endothelial cell migration and proliferation (Table 4), angiogenic growth factors (Table 5), matrix proteins on the endothelial cell surface, such as integrins (Table 6), copper (Table 7) and inhibitors with unique mechanisms (Table 8).

Table 3. — Angiogenesis Inhibitors in Current Clinical Trials: Protease Inhibitors

Drug	Mechanism	Sponsor	Trial
Marimastat	Synthetic matrix metalloprotease inhibitor (MMPI)	British Biotech	Phase III for cancer of breast, lung (non-small cell), pancreas, malignant glioma
Bay 12-9566	Synthetic MMPI and inhibitor tumor growth	Bayer	Phase III for carcinoma of lung, ovary, and pancreas
AG3340	Synthetic MMPI	Agouron/Warner-Lambert	Phase III for lung (NSCLC); phase III for prostate cancer
CGS 27023A	Synthetic MMPI	Novartis	Phase I/II
CGS 27023A	Synthetic MMPI	Novartis	Phase I/II
COL-3	Synthetic MMPI; Tetracycline derivative	Collagenex; NCI	Phase I
Æ-941 (Neovastat)	Naturally occurring MMPI	Æterna	Phase III for colon and NSCLC (to start in 1999)
BMS-275291	Synthetic MMPI	Bristol-Myers Squibb	Phase I
Penicillamine	Urokinase inhibitor	NCI-NABTT; commercially available	Phase II for glioblastoma

**Table 4. — Angiogenesis Inhibitors in Current Clinical Trials:  
Direct Inhibitors of Endothelial Cell Proliferation/Migration**

Drug	Mechanism	Sponsor	Trial
TNP-470 (fumagillin derivative)	Inhibits endothelial cell growth	TAP Pharmaceuticals	TAP Pharmaceuticals lymphomas, and acute leukemias; phase II for advanced, adult, solid tumors
Squalamine	Inhibits sodium-hydrogen exchanger, NIHE3	Magainin	Phase III for lung (NSCLC); phase III for prostate cancer
Combretastatin	Induction of apoptosis in proliferating endothelial cells	Oxigene	Phase I; phase II to start late 1999
Endostatin	Inhibition of endothelial cells	EntreMed	Phase I solid tumor to start late 1999
Penicillamine	Blocks endothelial cell migration and proliferation	NCI – NABTT; commercially available	Phase II for glioblastoma
Farnesyl Transferase Inhibitor (FTI)	Blocks endothelial cell migration and proliferation	NCI – NABTT	Phase I for solid tumors and glioblastoma to start 1999 – 2000
-L-778,123		Merck	
-SCH66336		Schering-Plough	
-R115777		Janssen	

**Table 5. — Angiogenesis Inhibitors in Current Clinical Trials:  
Antagonists of Angiogenic Growth Factors**

Drug	Mechanism	Sponsor	Trial
Anti-VEGF Antibody	Monoclonal antibody that inactivates VEGF	Genentech	Phase II/III for cancers of the lung, breast, prostate, colorectal, and renal
Thalidomide	Blocks activity of angiogenic growth factors (bFGF, VEGF, TNF-alpha)	Celgene	Phase II for Kaposi's sarcoma, glioblastoma, cancer of the prostate, lung, and breast
SU5416	Blocks VEGF receptor (Flk-1/KDR) signaling (tyrosine kinase)	Sugen-NCI	Phase I/II for Kaposi's sarcoma; phase I/II for metastatic colorectal cancer; phase I/II for advanced malignancies
Ribozyme (Angiozyme)	Attenuates mRNA of VEGF receptors	Ribozyme Pharmaceuticals, Inc	Phase Ia studies completed to establish pharmacokinetics
SU6668	Blocks VEGF, bFGF, and PDGF receptor signaling	Sugen	Phase I for advanced cancer
PTK787/ZK22584	Blocks VEGF receptor signaling	Novartis	Phase I for advanced cancers (Germany and UK); phase I for glioblastoma and Kaposi's sarcoma; phase I/II for von Hippel-Lindau disease
Interferon-alpha	Inhibition of bFGF and VEGF production	Commercially available	Phase II/III
Interferon-alpha	Inhibition of bFGF and VEGF production	Commercially available	Phase II/III
Suramin	Blocks binding of growth factor to its receptor	NCI – NABTT	Phase II for glioblastoma



**Table 6. — Angiogenesis Inhibitors in Current Clinical Trials: Drugs That Inhibit Endothelial-Specific Integrin/Survival Signaling**

Drug	Mechanism	Sponsor	Trial
Vitaxin	Antibody to alpha-v-beta3 integrin present on endothelial cell surface	Ixsys	Phase II for leiomyosarcoma
EMD121974	Small molecule blocker of integrin present on endothelial cell surface	Merck KGaA	Phase I/II for Kaposi's sarcoma, brain tumors, and solid tumors (to begin in 1999)

**Table 7. — Angiogenesis Inhibitors in Current Clinical Trials: Chelators of Copper**

Drug	Mechanism	Sponsor	Trial
Penicillamine	Sulfhydryl group binds copper; clears copper through urinary excretion	NCI-NABTT Brain Tumor Consortium	Phase II for glioblastoma
Tetrathiomolybdate	Thiol groups tightly bind copper, inactivate copper available to tumor	University of Michigan Cancer Center	Phase I/II trial for advanced metastatic cancer, multiple tumor types
Captopril	Chelates copper and zinc; also, inhibitor of MMP and angiotensin converting enzyme	Northwestern University	Phase I/II clinical trial

**Table 8. — Angiogenesis Inhibitors in Current Clinical Trials: Angiogenesis Antagonists with Distinct Mechanisms**

Drug	Mechanism	Sponsor	Trial
CAI	Inhibitor of calcium influx	NCI	Phase II/III for ovarian, non-small cell lung, and renal cell cancers
ABT-627	Endothelin receptor antagonist	Abbott/NCI	Phase I, refractory prostate and other malignancies; phase II, glioblastoma and prostate
CM101/ZD0101	Group B Strep toxin that selectively disrupts proliferating endothelium by interaction with the (CM201) receptor	CarboMed/Zeneca	Phase I trials completed; phase II trials to start
Interleukin-12	Induction of interferon-gamma	M.D. Anderson Cancer Center/Temple University	Phase I trials for ovarian, renal cell, melanoma, and gastrointestinal cancers; phase I/II for Kaposi's sarcoma, and solid tumors
	Down-regulation of IL-10	Temple University	
	Inhibits production of matrix metalloproteases	Genetics Institute	
	Induction of IP-10	Hoffman LaRoche	
IM862	Blocks production of VEGF and bFGF; increases production of the inhibitor IL-12	Cytran	Phase III for AIDS-related Kaposi's sarcoma

FN-145156E	Blocks angiogenesis induced by Tat protein	Pharmacia and Upjohn	Phase I trial for solid tumors; phase II planned
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## Angiogenic Inhibitors in Current Clinical Trials

### *Protease Inhibitors*

Marimastat (BB-2516) is the first matrix metalloproteinase (MMP) inhibitor to be tested in humans.<sup>44,45</sup> Given orally, marimastat has excellent bioavailability. Phase I studies involved healthy volunteers who received short courses of marimastat; these were well tolerated. Symptoms experienced by many patients with various malignancies included severe joint and muscle pain that were debilitating in more than 60% of patients at doses greater than 50 mg bid. These symptoms were reversible when the drug was stopped. The incidence of fibromyalgia has been decreased by using 10 mg bid of marimastat, the dose used in current studies. Phase II studies involved the use of serum tumor markers as surrogate indicators of antitumor activity. Six studies in patients with colorectal, ovarian, and prostate cancer have been completed. Pooled analysis demonstrates a dose-dependent biological effect; 58% of patients respond at doses greater than 50 mg bid. Alterations of tumor markers correlated with increased survival. Small phase II studies suggest potential activity in pancreatic and gastric cancer. These studies also demonstrate the safety of combining cytotoxic chemotherapeutic agents with marimastat. Ongoing phase III studies are investigating the effects of marimastat in addition to chemotherapy in the treatment of small cell lung cancer, gastric, and pancreatic carcinoma.

Because marimastat inhibits the growth and spread of pancreatic cancer in animal models, Rosemurgy et al<sup>46</sup> prospectively studied 64 patients with advanced carcinoma of the pancreas in whom standard treatments failed. Marimastat was well tolerated. Musculoskeletal pain, stiffness, and tenderness emerged as dose-limiting toxicities. The overall median survival was 160 days, with a 1-year survival of 21%. Marimastat was associated with an acceptable toxicity profile, and the preliminary data suggest that long-term oral administration is feasible and safe. Doses of 5, 10, and 25 mg twice daily were identified as the optimal doses to be tested in larger randomized studies.<sup>46</sup>

**BAY 12-9566** is a selective, nonpeptidic oral inhibitor of MMP-2 and MMP-9 angiosuppressive compound. Expression of these two MMPs correlates with a poor prognosis for cancers of the ovary, lung, breast, and colon.<sup>47</sup> Clinical pharmacology in phase I studies<sup>48</sup> shows that a dose level of 800 mg po bid produced steady-state plasma levels (>100 mg/L) sufficient for preclinical activity, and it shows evidence of antitumor activity in humans, without musculoskeletal toxicities. In 18 of 29 patients treated for an average of 2.5 months, the disease was stabilized.<sup>48</sup> The main toxicities were transient dose-related thrombocytopenia and transaminase elevations.

A phase I study determined that BAY 12-9566 could be given safely with standard doses of paclitaxel and the combination of paclitaxel and carboplatin.<sup>47</sup> Currently, BAY 12-9566 is in phase III clinical trials for small cell carcinoma of the lung, non-small cell carcinoma of the lung, metastatic pancreatic carcinoma, and recurrent ovarian carcinoma.<sup>48</sup>

**AG 3340** is a novel, synthetic, rationally designed, oral, selective MMP inhibitor that is potent with Ki values against specific gelatinases in the low picomolar range. Because it is not a broad-spectrum MMP inhibitor, it is believed that its clinical toxicity will be limited. It is effective to inhibit tumor growth and angiogenesis in a variety of preclinical models including cancers of the colon, lung (NSCLC), and breast<sup>49</sup> as well as human U87 gliomas.<sup>50</sup> The combination of AG3340 and several chemotherapeutic agents have been shown to have synergistic effects. Phase I studies, in combination with paclitaxel and carboplatin, showed the combination to be safe and well tolerated.<sup>51</sup> The drug is currently in phase III clinical trials for patients with non-small cell lung cancer in combination with paclitaxel and carboplatin, as well as in advanced hormone refractory prostate cancer in combination with mitoxantrone.<sup>52,53</sup> The most common side effects are in the musculoskeletal system (occasional joint stiffness, swelling, and limitation of mobility) and fatigue. These side effects cease after the treatment is stopped. Because a reduced rate of growth associated with MMP inhibition may extend beyond the time of

disease progression, patients enrolled in these studies may continue therapy with placebo tablets in a blinded manner during second- and third-line therapies.<sup>53</sup>

**Neovastat (AE-941)** is a naturally occurring (shark cartilage extract), oral agent that shows angiosuppressive and anti-MMP activities in vitro and in the chorioallantoic membrane (CAM) assay.<sup>54</sup> It is currently in phase I/II clinical trials for refractory lung, breast, and prostate cancers. In clinical studies with more than 540 patients, there were no serious clinical toxicity or laboratory abnormalities. The main side effects are gastrointestinal (nausea, vomiting), but overall, Neovastat demonstrates a favorable toxicity profile in a wide several clinical studies.<sup>54,55</sup> It will be evaluated by the NCI for pivotal phase III trials for treatment of colon and lung carcinomas.

### *Inhibitors of Endothelial Cell Migration and Proliferation*

**TNP-470 (fumagillin analog)** is a derivative of fumagillin, a naturally occurring inhibitor of angiogenesis and tumor growth.<sup>56-58</sup> TNP-470 is not cytotoxic in vitro, but it inhibits bFGF- and PDGF-stimulated endothelial proliferation. In vivo, TNP-470 inhibits angiogenesis in the chick CAM assay, the corneal assay, and the surgically implanted Matrigel assay. In mice, TNP-470 reduces the concentration of bFGF in surgically induced wounds; it does not alter the repair process if given before or 5 days after wounding.<sup>57</sup>

To date, TNP-470 has been used in more than 188 patients in phase 1 trials and in 127 patients in phase II studies.<sup>6</sup> At doses of 70 mg/m<sup>2</sup>, dose-related, reversible CNS toxicities (encephalopathy, dizziness, ataxia, and imbalance) occasionally occur. Fatigue, a nonspecific symptom, is not dose-related. Current phase II trials are ongoing for patients with glioblastoma (50 mg/m<sup>2</sup>), stage II/III pancreatic cancer (60 mg/m<sup>2</sup>), cervical cancer (60 mg/m<sup>2</sup>), and renal cell carcinoma (60 mg/m<sup>2</sup>). TNP-470 shows evidence of efficacy in the treatment of cervical cancer<sup>59</sup> and Kaposi's sarcoma.<sup>60</sup>

**Squalamine (MSI 1256 F)**, originally derived from the liver of a dogfish shark, is a novel noncytotoxic aminosterol with potent antiangiogenic properties in vivo and in vitro.<sup>61</sup> Squalamine prevents the neovascularization of tumors by suppression of endothelial cell migration and proliferation. A phase I study was performed to evaluate the toxicity at doses ranging from 6 to 255 mg/m<sup>2</sup> per day. Steady-state concentrations in patients treated with 24 to 66 mg/m<sup>2</sup> per day resulted in plasma concentrations of 0.37 to 0.51 mg/mL, approximating those required for antiangiogenic effects in vitro. There were no serious (NCI grade 3 or 4) toxicities in a study of 12 patients.<sup>62</sup>

A second phase I study determined the maximum tolerated dose-limiting toxicity and pharmacokinetics when given as a 120-hour continuous intravenous infusion to patients with advanced cancer.<sup>63</sup> Dose-limiting toxicities were grade 3 elevations of transaminase (2 of 16 patients) at a dosage of 538 mg/m<sup>2</sup>. Other toxicities noted were a grade 2-3 (fatigue), and a grade 1-2 (nausea, anorexia, or myalgia with numbness). Steady-state concentrations (150 to 300 ng/mL) were reached within 24 hours at doses of 6 to 12 mg/m<sup>2</sup> per day. Fourteen of 16 patients experienced disease progression after two to nine courses of treatment. Two patients continue to receive treatment. There was no objective tumor response in patients with advanced cancer of the lung (3), ovary (3), melanoma (2), breast (2), pancreas (1), colorectal (1), sarcoma (1), and cholangiocarcinoma (1). Patient accrual is ongoing to define the optimal dose and duration of treatment for phase I/II trials of squalamine in combination with cytotoxic chemotherapy.

**Combretastatins** are small organic molecules found in the bark of the African bush willow, the *Combretum Caffrum*. Combretastatins not only suppress proliferating endothelium, but also specifically target tumor endothelium. The combretastatin A-4 prodrug is a derivative of combretastatin, which is activated by a phosphatase selectively amplified in proliferating endothelial cells. Combretastatin A-4 induces apoptosis in human endothelial cells.<sup>64</sup> In tumor-bearing mice, combretastatin A-4 significantly enhanced the antitumor effects of radiation therapy.<sup>65</sup>

**Endostatin** is a 20kDa C-terminal fragment of collagen XVIII discovered by O'Reilly et al.<sup>5</sup> Zinc-binding of endostatin is essential for its antiangiogenic activity.<sup>66</sup> Endostatin specifically inhibits endothelial proliferation and potently inhibits angiogenesis and tumor growth. Primary tumors regress to dormant microscopic lesions. There is no known toxicity in animal models. Furthermore, the concept of dormancy therapy<sup>4</sup> is being extended to endostatin using cycles of therapy. Endostatin was given to mice bearing Lewis lung carcinoma, T241 fibrosarcoma, or B16F10 melanoma; treatment was stopped when tumors regressed. Tumors were then allowed

to regrow. Following resumption of endostatin, after multiple treatment cycles, the tumors did not reappear after discontinuation of therapy. Remarkably, no drug resistance occurs with endostatin or angiostatin.<sup>67,68</sup> When angiostatin and endostatin are combined, the tumors do not recur once treatment is suspended. Thus, antiangiogenesis therapy could represent first-line treatment.

There has been great anticipation among oncologists and their patients since the reports of angiostatin and endostatin appeared in the media.<sup>3</sup> The NCI staff acknowledge that they had difficulty confirming the results in their own laboratories, but when they visited the laboratory at Harvard and repeated the experiments, they found striking inhibition of Lewis lung carcinoma in mice. The NCI has concluded that there are significant differences in storage, handling, and purification techniques that can alter the activity of endostatin. Preliminary data of the NCI confirm that endostatin is safe and well tolerated in animals. Full-scale toxicology studies of endostatin are underway.

As an outgrowth of the laboratory studies, the NCI plans to initiate phase I trials in patients with solid tumors, including cancers of the lung, breast, colon, prostate, as well as lymphoma. The first phase I trials, with enrollments of 15 to 25 patients, are planned to start in 1999 at the M.D. Anderson Cancer Center and at the University of Wisconsin.

**Angiostatin** is a 38 kDa circulating, endogenous, antiangiogenic and antimetastatic protein.<sup>4,69,70</sup> Angiostatin binds ATP synthase on the surface of human endothelial cells,<sup>71</sup> induces apoptosis in endothelial cells<sup>72,73</sup> and tumor cells,<sup>74</sup> and inhibits endothelial cell migration and tubule formation, but it does not affect growth-factor-induced signal transduction.<sup>73</sup> Furthermore, it activates the focal adhesion kinase, suggesting that it subverts the formation of endothelial cells adhesion plaques.<sup>73</sup> Expression analysis in angiostatin-treated tumors indicates a decrease in mRNA expression for VEGF and bFGF.<sup>74</sup> Angiostatin inhibits matrix-enhanced plasminogen activation to account, in part, for its angiosuppressive and anti-invasive properties.<sup>75</sup> Of great interest, angiostatin is generated by free sulfhydryl donors (eg, D-penicillamine and captopril) that may explain, in part, their angiosuppressive properties.<sup>70</sup>

As a paradigm for potential clinical use, angiostatin is of interest for several reasons. First, it is highly synergistic with endostatin, suggesting that combination antiangiogenesis strategies may be more effective than monotherapy with a single agent. Second, like endostatin, it appears to be effective at specific stages of carcinogenesis.<sup>22</sup> Third, it is synergistic with ionizing radiation in multiple tumor models, supporting the concept that angiosuppression will potentiate radiation therapy.<sup>76-78</sup> Fourth, angiostatin may be of value in treating vascular malignant gliomas, either by systemic administration<sup>74</sup> or by using retroviral or adenoviral vectors for gene-based therapies.<sup>79</sup>

Angiostatin is not available for human studies at this time. EntreMed Corp (Rockville, Md) is working with the NCI to bring angiostatin to the clinic for initial testing. Bristol-Myers Squibb Co (New York, NY) announced early in 1999 plans to discontinue its direct involvement to bring angiostatin to the clinic, largely due to practical difficulties of manufacturing, stability of the molecule, homogeneity, and purity of batches.

**Penicillamine** is a low-molecular-weight, antiangiogenic, copper-chelating molecule discovered to be an effective inhibitor of intracerebral glioma growth, invasiveness, and angiogenesis.<sup>24,25,39,80</sup> Penicillamine in vitro is a direct inhibitor of endothelial cell proliferation and migration in clinically relevant concentrations.<sup>23,81</sup> Penicillamine has multiple functions in addition to copper chelation that could account for its antiangiogenic activity (Table 9). For example, Gross et al<sup>39</sup> discovered that penicillamine is a dose-dependent inhibitor of urokinase-type plasminogen activator. Penicillamine by itself was found to inhibit brain tumor growth, but the inhibition was markedly enhanced when combined with copper depletion<sup>24</sup> — another example of synergistic combination antiangiogenesis therapy.

Table 9. — Angiosuppressive Mechanisms of Penicillamine and Copper Reduction		
Steps in the Angiogenesis	Pathway	Copper Reduction
Endothelial cell (EC) migration	Blocks EC migration	Blocks copper-stimulation of EC migration

Endothelial cell proliferation	Blocks EC proliferation	Blocks copper-stimulation of EC proliferation
Collagen synthesis and cross-linking	Inhibits (separate from copper defect)	Inhibits (separate from penicillamine)
Growth factor binding factors (eg, bFGF, VEGF)	—	Copper is a cofactor of heparin and copper-binding growth
Growth factor function	—	Reversibly activates or suppresses growth factor-induced angiogenesis dependent on level of copper
Protease activity	Dose-dependent inhibitor of uPA, tPA, gelatinase B activity; stabilizes TIMP	—
Angiostatin	Converts plasminogen (inert) to angiostatin (inhibitor) sulfhydryl donor	—
Effect on tumor growth as monotherapy	Mild inhibition	Mild inhibition
Effect on tumor growth as combination (penicillamine + copper reduction)	Strong inhibition	Strong inhibition

Farnesyl Transferase Inhibitors (FTIs): Activation of the *ras* oncogene is an important pathway to stimulate angiogenesis.<sup>20,21,82-84</sup> Furthermore, the tumorigenicity of the *ras* oncogene may be VEGF dependent.<sup>85</sup> At our institute, we tested the hypothesis that inhibitors of the enzymes farnesyl transferase and geranylgeranyl transferase would block angiogenesis in vitro.<sup>86</sup> Not only did FTI-277 and GGTI-298 produce a dose-dependent inhibition of glioma cell growth, but also the inhibition of *ras* and related G-proteins directly inhibited endothelial cell proliferation. Thus, the FTI and GGTI compounds, in addition to blocking the proliferation of glioma cells directly, may also function as antiangiogenesis compounds.<sup>86</sup>

Other inhibitors of farnesyl transferase are being evaluated in clinical trials. These compounds could function in part as angiogenesis inhibitors. L-778,123 (Merck and Co, Inc, Whitehouse Station, NJ) was tested as a continuous 7-day infusion every 3 weeks in a phase I clinical trial.<sup>87</sup> Sixteen patients received 35 courses at dose levels between 35 and 560 mg/m<sup>2</sup> per day. There were no serious drug related toxicities; mild to moderate nausea and vomiting and fatigue were noted in a few patients. Pharmacokinetic studies showed steady-state concentrations were achieved in the plasma within 3 hours. At a dose of 280 mg/m<sup>2</sup> per day, serum steady-state concentrations ranged from 3 to 5 mM, capable of significantly inhibiting the processing of *Ras* and the growth of tumors with *ras* mutations in preclinical models. Inhibition of farnesylation of the marker protein, hDJ2, in peripheral blood mononuclear cells paralleled plasma L-778,123 concentrations. The maximal inhibition was achieved on days 4 and 8 and returned to pretreatment levels by day 17. Taken together, the results indicate that L-778,123 is well tolerated at concentrations that produce relevant biological effects in preclinical studies and inhibit protein farnesylation in vivo.<sup>87</sup>

SCH66336 (Schering-Plough Corp, Madison, NJ) is an oral FTI that has broad antitumor activity in preclinical studies.<sup>88-90</sup> H-, K-, and N-*ras*, most G-proteins, rho B, pre-laminin A, and many other proteins require the posttranslational addition of the isoprenoid farnesyl group (or the related geranylgeranyl group) for biological activity. Daily administration in 24 patients at levels of 25 to 300 mg twice daily (bid) showed a maximal tolerated dose of 200 mg bid.<sup>88,90</sup> Reversible toxicities include fatigue, anorexia, nausea, vomiting, and diarrhea.<sup>88-90</sup> Further clinical testing is underway.<sup>88</sup>

A phase I trial of an oral R115777 (Janssen Pharmaceutica, Inc, Titusville, NJ) in 12 patients with refractory solid

tumors in doses of 60 to 420 mg/m<sup>2</sup>. Myelosuppression is the main dose-limiting toxicity using this schedule. The estimated maximal tolerated dose is 240 mg/m<sup>2</sup> twice daily. Plasma levels of R115777 in the tolerable dose range are similar to effective in vitro concentrations.<sup>91</sup>

Phase II clinical trials of FTIs for glioblastoma and other malignancies, sponsored in part by the NCI, are in the planning stage.

### *Antagonists of Angiogenic Growth Factors*

**Anti-VEGF Antibody:** VEGF is a critically important angiogenic growth factor. In animal models, an anti-VEGF monoclonal antibody inhibits tumor growth.<sup>92</sup> A phase I trial<sup>93</sup> of anti-VEGF antibody in 25 patients with metastatic cancer in doses ranging from 0.1 to 10.0 mg/kg intravenously over 90 minutes showed that the anti-VEGF antibody was well tolerated without grade 3 or 4 toxicities. There was a  $\geq 20\%$  incidence of grade 1 or 2 adverse events including headache, asthenia, fever, nausea or vomiting, arthralgias, cough, dyspnea, and rash.<sup>93</sup> Although there were no objective responses, a patient with renal cell carcinoma had a 39% reduction in tumor and 13 (52%) of 25 patients had stable disease after 10 weeks. The anti-VEGF dosing resulted in anticipated decreases in free VEGF concentrations. No patients developed an antibody to anti-VEGF antibody.<sup>93</sup>

A second phase I trial was performed to evaluate safety/toxicity using the antibody to VEGF in combination with cytotoxic chemotherapy; this trial was conducted due to antitumor synergisms in animal models when both were applied.<sup>94</sup> The VEGF antibody, given once per week for 8 weeks, could be safely combined with chemotherapy (doxorubicin, carboplatin, or 5-fluorouracil with leucovorin). Neither the pharmacology of the antibody nor the clearance of the chemotherapeutic agent was affected. There were no identifiable late toxicities associated with long-term therapy. Three of 12 patients showed tumor regression, and two patients continue to receive chemotherapy plus the VEGF antibody 11 to 12 months after the start of the study. Randomized trials to determine efficacy are planned.<sup>94</sup>

**Thalidomide:** Thalidomide is a potent teratogen and sedative that inhibits angiogenesis induced by bFGF<sup>95</sup> and VEGF.<sup>37</sup> It is effective orally and has been found useful in the treatment of malignant recurrent gliomas, especially when given in combination with a cytotoxic chemotherapeutic drug such as carboplatin.<sup>96</sup>

In a phase I/II trial,<sup>96</sup> 71 patients with recurrent glioblastoma used a maximal tolerated dose of 300 mg/m<sup>2</sup>. At this dosage, 46 patients were evaluable for efficacy. Thirty-three patients had responses (5 partial, 28 stable disease), and 13 had progressive disease. The median survival was 40 weeks. The median response duration was 24 weeks. Toxicity attributed to thalidomide included drowsiness and constipation. The combination of carboplatin and thalidomide appears more effective than either agent alone for recurrent glioblastoma.<sup>96</sup>

In a phase II study of 28 patients with metastatic breast cancer, two dose levels (200 mg vs 800 mg per day) were compared for toxicity and efficacy.<sup>97</sup> At the 200-mg level, two patients had stable disease and tolerable side effects after 8 weeks of treatment. At the 800-mg dose, side effects included somnolence and peripheral neuropathy. Less severe side effects that did not require dose reduction included fatigue, dry mouth, dizziness, nausea, anorexia, headaches, skin rash, and neutropenia. Overall, thalidomide as a single agent was not effective for this patient population.

**Sugen 5416:** SU 5416 is a novel synthetic compound, a specific VEGF receptor (Flk-1) antagonist that decreases VEGF-stimulated Flk-1 phosphorylation.<sup>98</sup> Because it is a specific angiogenesis antagonist, SU5416 does not directly inhibit tumor cells in vitro. However, SU5416 shows broad antitumor efficacy in subcutaneously implanted tumor xenografts in athymic mice.<sup>98</sup> In 63 patients with a variety of advanced cancers, their disease was generally stable after six months of treatment. The tumors included Kaposi's sarcoma, non-small cell carcinoma of the lung, and colorectal, renal cell, adenoid cystic, and basal cell carcinomas.<sup>99</sup> The pharmacokinetics indicated extensive tissue penetration and dose-independent clearance.<sup>99</sup> The study is being expanded to treat at the recommended optimal dosage of 145 mg/m<sup>2</sup>.<sup>100</sup>

**Antiangiogenic Ribozyme:** The pharmacology and toxicology of an antiangiogenic ribozyme is being developed. The ribozyme Angiozyme (Ribozyme Pharmaceuticals, Inc, Boulder, Colo) inactivates mRNA for two VEGF receptors (Flt-1 and Flk-1 [KDR]), thereby disrupting the VEGF signaling pathway, inhibiting angiogenesis, and

suppressing tumor growth in preclinical models (Lewis lung metastases and colon carcinoma). The ribozyme is well tolerated in animals. Phase Ia trials in humans are completed, showing good pharmacokinetics with a half-life of >2 hours when given subcutaneously.<sup>101</sup>

**SU6668:** SU6668 is a potent, broad-spectrum tyrosine kinase inhibitor that combines both antiangiogenic and antitumor properties.<sup>102</sup> Given orally, there are measurable plasma levels for 24 hours to support once-daily oral schedule. Preliminary data show that analogs of SU6668 (and SU5416) inhibit glioma cell growth and the production of matrix metalloproteases associated with tumor growth and angiogenesis and that SU5402 (an analog of SU5416 and SU6668) inhibits the formation of matrix metalloproteases produced by cancer cells of the prostate.

**Interferon Alpha:** Interferon-alpha2a (IFN-alpha2a) was the first angiogenesis inhibitor to be used in clinical trials and was effective in children for the treatment of life-threatening hemangiomas.<sup>103-105</sup> However, because of the occurrence of severe neurological toxicity (spastic diplegia), IFN-alpha must be used with caution for treatment of hemangiomas.<sup>105</sup>

Another clinical indication has been giant-cell tumor of the bone.<sup>106,107</sup> Kaban et al<sup>106</sup> reported the dramatic regression of a large, rapidly growing, recurrent giant-cell tumor of the mandible. The angiogenic protein bFGF was initially elevated in the urine, but after one year of treatment, the bone tumor regressed and disappeared, the urinary bFGF fell to normal levels, and the mandible regenerated. In a series of 10 patients with unresectable or metastatic giant-cell tumor treated with interferon-alpha2b,<sup>107</sup> five patients had major responses including three of five patients with pulmonary metastases. Responses occurred slowly and continued after ceasing therapy. In one case, disease progressed throughout 6 months of therapy and only then started to regress. The median time to maximal response was 3.1 years. Four patients had rapidly progressive disease, and treatment was discontinued. One additional patient had stable disease for 7 months after discontinuation of therapy but then progressed.

These reports demonstrate some of the principles that are important in designing clinical trials of therapeutic angiosuppression for malignant disease. (1) Tumors that express bFGF (or other angiogenic factors such as VEGF) may successfully respond to IFN-alpha or other agents that suppress the expression of bFGF (or VEGF, etc), (2) angiosuppressive therapy, given uninterrupted for one year, can be safe and effective, (3) treatment can be continued for one or more years without the development of drug resistance, and (4) angiosuppressive therapy, in contrast to cytotoxic chemotherapy, requires prolonged treatment and follow-up. Responses can continue and may become noticeable after discontinuing treatment.

**Suramin:** Suramin is the prototype of a growth factor antagonist and discovered to inhibit the mitogenic action in vitro of numerous growth factors. Suramin inhibits multiple molecular control points of angiogenesis, including the production of urokinase-type plasminogen activator.<sup>40</sup> Suramin is a polysulfonated molecule and interferes with the binding of the growth factor to its receptor.<sup>108</sup> Suramin inhibits endothelial cell proliferation and migration (Fig 3) and is a dose-related inhibitor of angiogenesis in the chick CAM assay (Fig 4). It was also shown to be effective in the brains of animals where suramin inhibited the proliferation of both glioma and endothelial cells.<sup>40</sup> In animals, it produced significant cerebral hemorrhages, possibly because of its heparin-like properties. Based on its angiosuppressive and antiglioma properties, suramin was evaluated by the NABTT Consortium. Using a dosing regimen similar to one found effective for prostate cancer, 12 patients with malignant gliomas were treated.<sup>109</sup> Ten of the 12 patients were diagnosed with glioblastoma; 11 of 12 patients had received prior nitrosoureas. The actual drug-related toxicities were mild and reversible.<sup>109</sup>

The results underscore the challenges to evaluate clinical responses modified by cytostatic angiosuppressive molecules in contrast to classic cytostatic chemotherapeutic agents. One patient removed from the study due to "progression" at 10 weeks had a partial response 7 months later and remains free of dexamethasone with a Karnofsky Performance Status scale of 100%. This patient did not receive post-suramin antineoplastic therapy. Another patient showed disease stabilization and lived for 2.2 years without any other therapy. Pharmacokinetics were available for 11 patients. The target serum concentrations of 100 to 300 µg/mL were achieved, and 9 of the 11 patients were on p450-inducing anticonvulsants.<sup>109</sup> In light of these findings, the NABTT CNS Consortium plans a further study of suramin for newly diagnosed malignant glioma patients in combination with radiation therapy. The angiogenesis inhibitor will be evaluated in patients expected to live sufficiently long to benefit from the cytostatic properties, and the primary end-point will be survival in contrast to tumor volumetric reduction.<sup>109</sup>

### *Inhibitors of Endothelial-Specific Integrin/Survival Signaling*

**Integrin Antagonists - Vitaxin:** Cheresh<sup>110</sup> discovered that the alpha-v-beta3 integrin is a critically important adhesion molecule in the regulation of angiogenesis<sup>110</sup> and that it promotes endothelial and tumor cell survival.<sup>111</sup> The vascular integrin has been used as a prognostic biomarker in breast cancer<sup>112</sup> and can be used for tumor detection using magnetic resonance imaging (MRI).<sup>113</sup> The LM609 antibody to the integrin (Vitaxin) has moved from phase I trials to phase II studies. Vitaxin has been proven to be safe and has led to the stabilization of disease in most of the patients treated thus far.<sup>114</sup>

### *Copper Antagonists/Chelators*

If angiogenesis controls cancer growth, what controls angiogenesis? We are learning that the copper status is critical to the function of the angiogenic growth factors (Fig 6).

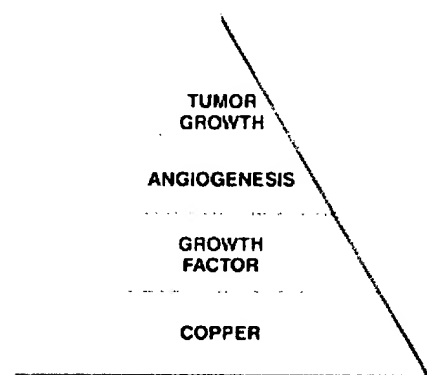


Fig 6. — Hierarchy of control mechanisms for tumor growth. The concept of tumor growth driven by angiogenesis is well accepted,<sup>7</sup> but what drives angiogenesis? To translate from the molecular or ionic level to the clinical level involves "vertical reasoning."<sup>185</sup> Based on several lines of evidence, it is reasonable to hypothesize that angiogenesis is dependent on the copper status.

Recent work supports our hypothesis that copper mediates the "switch" (Fig 7) of the normally quiescent ( $G_0$ ) endothelium into a proliferative state by activation of the angiogenic growth factors.<sup>115</sup> We observed that copper reduction, using a low-copper diet and a chelator of copper (penicillamine), inhibits the angiogenic activity of four structurally diverse angiogenic factors and cytokines (Fig 8).



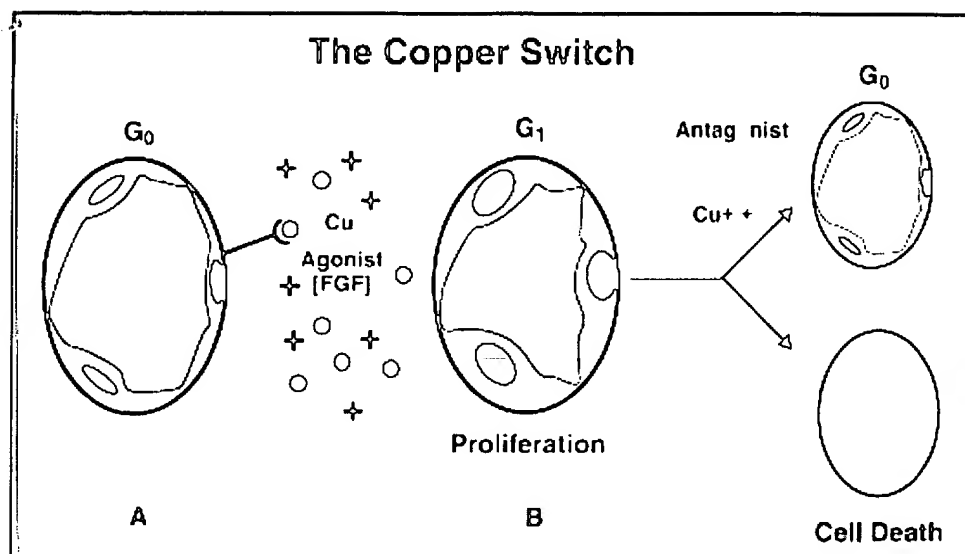


Fig 7. — Hypothetical scheme of a proposed "copper switch" that turns angiogenesis "on" (copper-sufficient) or "off" (copper deficient). Copper acts as an obligatory cofactor and is permissive to the angiogenic activator. Copper reduction blocks angiogenesis by "switching" endothelial cell into apoptosis pathway or quiescence ( $G_0$ ).

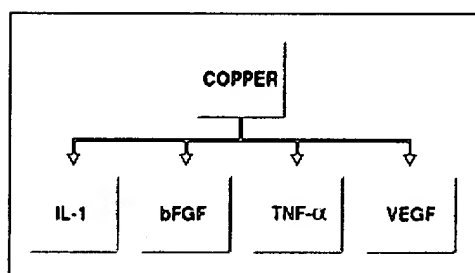


Fig 8. — Four structurally diverse angiogenesis cytokines/growth factors are inactivated by copper withdrawal. From Brem et al.<sup>115</sup>

The angiogenic activity of bFGF, vascular endothelial growth factor (VEGF), TNF- $\alpha$ , and IL-1 were found to be copper dependent.<sup>115</sup> Furthermore, copper repletion switches angiogenesis back "on" when a copper-sufficient diet is restored, providing evidence for a novel, physiologic, and metabolic control pathway of angiogenesis. This mechanism could explain the inhibition of angiogenesis observed in the brains of animals by copper reduction.<sup>24,25</sup> These observations led to the clinical trial of penicillamine and a low copper diet for patients with glioblastoma (Table 10).

Table 10. — Summary of NABTT Clinical Trial, 97-04: Phase II Clinical Trial of Penicillamine and Reduction of Copper for Angiosuppressive Therapy of Newly Diagnosed Glioblastoma	
Sponsor	NCI (CTEP) and NABTT (New Approaches to Brain Tumor Therapy) Consortium
Rationale	Penicillamine
	(1) is a selective inhibitor of angiogenesis,
	(2) forms disulfide bonds that inactivates vascular growth factors,
	(3) contains a sulfhydryl group that converts plasminogen to angiostatin,

	(4) chelates and excretes copper,
	(5) inhibits collagen crosslinking,
	(6) is a protease inhibitor, and
	(7) has a low-molecular weight, active in CNS.
	<b>Copper</b>
	(1) is an obligatory cofactor of angiogenesis,
	(2) stimulates endothelial cell migration and proliferation,
	(3) activates vascular growth factors, and
	(4) is required for collagen synthesis.
<b>Eligibility</b>	At least 18 years old
	No previous biological therapy, chemotherapy, or radiation therapy
	Must be on stable corticosteroid regimen for at least 1 week
	No allergy to penicillin
	Histologically confirmed glioblastoma with or without measurable (macroscopic) tumor
	Adequate baseline laboratory values
<b>Treatment</b>	Penicillamine (250 mg) is given orally over a 5-week escalation from 250 mg daily to 2 gm daily. Patients receive radiation therapy for 6 weeks beginning on the first day of penicillamine therapy. Patients also maintain a diet low in copper (0.5 mg/day) and receive vitamin B6 daily.
<b>End-points</b>	Primary end-point is time of survival. Secondary end-points are reduction of serum copper level, delay in time to progression, and reduction of tumor volume.
<b>Principal Investigator</b>	Steven Brem, MD, Moffitt Cancer Center, (813) 979-3063.

**Copper and Angiogenesis:** In an attempt to McAuslan isolate a peptide, endothelial stimulating growth factor, McAuslan and Reilly<sup>116</sup> noted a high concentration of copper salts. They postulated that copper was the "active principal" in angiogenesis. Copper, but not other trace metals, stimulated the directional migration of endothelial cells. More recently, copper was found to stimulate directly the in vitro proliferation of endothelial cells.<sup>117</sup> In a series of elegant experiments at the NCI, Gullino and co-workers<sup>118</sup> discovered that the availability of copper in vivo is critical to the initiation and development of angiogenesis. Using a low copper diet and penicillamine therapy, prostaglandin E-stimulated angiogenesis was suppressed. Diverse angiogenic molecules show high affinity for copper.<sup>27</sup> Copper-binding molecules (ceruloplasmin, heparin, and the tripeptide glycyl-histadyl-lysine) are non-angiogenic when free of copper, but they become angiogenic when bound to copper.<sup>119</sup>

**Copper and Cancer:** Copper metabolism is profoundly altered in neoplastic development in human cancer and in tumor-bearing animals.<sup>120,121</sup> Ceruloplasmin, the principal copper-transporting protein, increases four- to eight-fold during malignant progression, often before tumors become palpable; tumor regression returns ceruloplasmin levels to normal.<sup>122,123</sup> Serum copper levels correlate with tumor incidence, tumor burden, malignant progression, and recurrence in a variety of human cancers (Hodgkin's lymphoma, sarcoma, leukemia, and cancer of the cervix, breast, liver, and lung)<sup>124-127</sup> as well as brain tumors.<sup>128,129</sup> In preclinical tests, Yoshida et al<sup>129</sup> noted that penicillamine and copper reduction lowered the tissue levels of copper in the brain to nearly normal levels, reversing the copper toxicosis associated with a brain tumor.

**Tetrathiomolybdate:** Merajver et al<sup>130</sup> recently reported that copper depletion prevents the development of mammary cancer in HER2/neu+ transgenic mice that were given oral tetrathiomolybdate, a potent and nontoxic chelator copper. When tetrathiomolybdate was given to animals with large tumors, the tumors shrank. Immunohistochemical measurements of factor VIII and apoptosis were used to establish intermediate end-points of antiangiogenesis.<sup>130</sup> A phase I/II study of oral tetrathiomolybdate for patients with advanced metastatic cancers is encouraging. Preliminary results show that "in patients who survive long enough to become copper deficient,

which takes about three to four weeks, their tumors have stopped growing."<sup>131</sup>

**Captopril:** Captopril is an orally administered drug already in widespread use to treat nonmalignant disease. Yet, it can antagonize several steps in the angiogenesis cascade, resulting in decreased tumor growth.<sup>132,133</sup> Used commonly as an inhibitor of the angiotensin converting enzyme to treat hypertension, the sulfhydryl group enables captopril, like penicillamine, to convert plasminogen to angiostatin.<sup>70</sup> Captopril is known to be a chelator of copper,<sup>134,135</sup> or to function as a metalloprotease inhibitor.<sup>136</sup> There is anecdotal evidence that captopril, combined with urokinase, causes regression of an advanced refractory malignancy.

### *Angiogenic Inhibitors With Distinct Mechanisms*

**Carboxyamido-Triazole (CAI) - An Inhibitor of Calcium Influx:** CAI is an orally given, low-molecular-weight synthetic compound that inhibits calcium influx, endothelial cell proliferation, and neovascularization in physiologically attainable concentrations.<sup>6,137</sup> The long-term half-life of CAI permits a once-daily schedule. The inhibitory effects of cell-cell signaling, proliferation, and invasion, using CAI, are reversible — similar to other cytostatic agents.<sup>32</sup>

Phase I clinical trials of CAI showed a safe therapeutic window.<sup>32</sup> Disease stabilization was observed in patients heavily pretreated with advanced colorectal, pancreatic, renal cell, ovarian, and breast cancer.<sup>137,138</sup> The primary toxicity observed included gastrointestinal intolerance. Serious side effects that were reversible with drug discontinuation included retinal hyperemia and a concentration-dependent cerebellar ataxia possibly associated with cognitive dysfunction.<sup>32</sup> Phase II trials are planned for multiple primary disease sites, including patients with brain tumors.

A synergistic antiproliferative effect of CAI and paclitaxel (but not carboplatin) has been observed *in vitro* and is the basis for an ongoing clinical trial.<sup>6</sup>

**ABT-627 - An Endothelin Receptor Antagonist:** Endothelin-1 (ET-1) is a potent, vascular, smooth-muscle mitogen and vasoconstrictor; the expression of endothelin-1 correlates with tumor vascularity and malignancy of human astrocytomas.<sup>139</sup> Experimental data support a role for endothelin-1 in the pathophysiology of adenocarcinoma of the prostate<sup>140</sup> and the ovary.<sup>141</sup> The level of endothelin-1 in plasma is significantly elevated in patients with colorectal cancer and nearly double in patients with colorectal metastases to the liver.<sup>142</sup>

ABT-627 is a selective receptor antagonist of the ET<sub>A</sub> receptor. In a dose-escalation study of 26 patients with hormone refractory prostate cancer,<sup>143</sup> there were declines in the prostate-specific antigen and/or stabilization of the disease by computed tomography scan and bone scintigraphy in 19 patients (73%). There were no grade 3-4 toxicities. In 29 patients with refractory adenocarcinoma,<sup>144</sup> given dosages ranging from 10 to 75 mg/day, the toxicity was minimal. The main side effects were transient grade 2 headache (35%), rhinitis (91%), mild anorexia (35%), and fatigue (35%). Encouraging findings were early tumor marker changes and improvement in pain, and there were no measurable responses in a brief phase I trial.<sup>144</sup> Phase II trials are underway for treatment of prostate cancer. A phase II trial is planned for human glioblastoma using the NABTT Brain Tumor Consortium.

**CM101 - A Bacterial Toxin That Selectively Attacks Proliferating Vessels:** Hellerqvist and colleagues<sup>145</sup> discovered that the group B streptococcus toxin (CM101) interacts with receptors selectively on proliferating blood vessels but not on nonproliferating vessels. CM101 induces a complement-activated, cytokine-driven inflammatory reaction targeting a specific receptor, CM201, found in proliferating endothelium. Phase I trials for patients with advanced cancer have been completed with encouraging results.<sup>145,146</sup> The optimal dosage is 15 to 25 µg/kg every other week for 10 weeks. CM101 will be developed further by AstraZeneca, Inc, as ZDO101.

**Interleukin-12:** IL-12 is a multifunctional cytokine discovered to be an antiangiogenic agent<sup>147-149</sup> that enhances antitumor activity in preclinical models.<sup>150</sup> The antiangiogenic activity of IL-12 is linked to the IFN-gamma-induced IP-10 molecule. Phase I trials using the subcutaneous and intravenous routes have shown responses in patients with renal cell cancer and melanoma. Dose-limiting toxicities encountered at doses of  $\geq 1,000$  ng/kg, include leukopenia, stomatitis, and elevated transaminases. A phase I trial is underway to determine the maximum tolerated doses in patients with peritoneal carcinomatosis secondary to ovarian and gastrointestinal

malignancies.<sup>151</sup> Even at doses as low as 3 ng/kg, however, significant biological activity is observed, including modulation of angiogenesis-related molecules.<sup>151</sup>

**IM862 - A Peptide That Blocks Production of VEGF and bFGF and Stimulates IL-12 Production:** IM862 is a naturally occurring small peptide that inhibits angiogenesis in the CAM assay. The protein blocks production of VEGF and bFGF. It increases production of an immune-boosting cytokine and an inhibitor of angiogenesis, IL-12.<sup>152</sup> When given as intranasal drops to AIDS patients with Kaposi's sarcoma who were also taking protease inhibitors, there was a response rate of 37% (partial or complete remissions). Adverse effects to IM862 are mild and limited to transient headaches, fatigue, tingling, and nausea.<sup>152</sup>

**PNU145156E - A Suramin Analog That Blocks Angiogenesis Induced by the Tat Protein:** PNU145156E is a sulfonated distamycin A derivative and a suramin analog that demonstrates antiangiogenic and antitumor effects in preclinical models.<sup>153</sup> It blocks the angiogenesis induced by the Tat protein.<sup>154</sup> The recommended dose for phase II studies is 840 mg/m<sup>2</sup>. Like suramin, it has a long half-life. In a phase I trial of 29 patients at dose levels of 100 to 1,050 mg/m<sup>2</sup> given at 1-hour intravenous infusions every 6 weeks, there were no tumor responses observed nor any changes in serum levels of bFGF or VEGF.<sup>155</sup>

## Emerging Concepts in Angiogenesis Research

### *Gene Therapies for Antiangiogenesis*

Among the most promising of exciting new gene therapies are the regulators of angiogenesis.<sup>156</sup> For example, it has been demonstrated that the transfection of antisense-VEGF-cDNA results in down-regulation of the endogenous VEGF and suppresses the ability of glioma cells to form tumors in mice.<sup>156</sup> In addition, the transfer of antisense VEGF to U87 malignant glioma cells in vivo, by using an adenovirus (Ad5CMV-alpha), inhibits tumor growth.<sup>157</sup> Use of the adenovirus-mediated wild-type p53 gene transfer to human colon carcinoma cells results in decreased levels of VEGF and decreased angiogenesis in vivo.<sup>158</sup> Direct injection of the endostatin gene into mouse muscle is followed by local expression of endostatin and detectable levels of endostatin secreted into the bloodstream for up to 2 weeks following a single injection.<sup>159</sup>

Gene transfer of a cDNA coding for mouse angiostatin into murine fibrosarcoma cells suppresses primary and metastatic tumor growth in vivo. The transfected metastases are maintained in a prolonged dormancy state where high cell proliferation is balanced by apoptosis. The metastases remain avascular for several months.<sup>37</sup>

Cationic liposome-DNA complex-based intravenous gene delivery targets gene expression to vascular endothelial cells, macrophages, and tumor cells.<sup>37</sup> Cationic liposome-DNA complex-based intravenous delivery of the p53 gene was as effective as the angiostatin gene in reducing tumor metastasis and angiogenesis<sup>37</sup> and induced the expression of the thrombospondin gene. Combination delivery of multiple genes is as effective as a single gene, suggesting that the genes tested (p53, granulocyte-macrophage stimulating factor, and angiostatin) each inhibit a common angiogenesis pathway.

Recombinant adenovirus directing the secretion of an antagonist of cell-associated urokinase, blocking the attachment of uPA to its receptor (uPAR), was recently shown to control local malignant tumor growth and angiogenesis as well as distant metastases of the Lewis lung carcinoma or human colon carcinoma xenograft when given systemically or locally by injection into the tumor.<sup>160</sup>

A promising approach is the use of retroviruses encoded with a deficient VEGF receptor-2 (VEGFR-2). Survival time of rats with intracerebral tumors was significantly prolonged in a dose-dependent manner when the retroviruses carrying the VEGFR-2 were co-transplanted with tumor cells. Controls, cells without virus or the supernatant, failed to show an effect on survival. Tumors showed signs of impaired angiogenesis. Retrovirus-mediated gene transfer was found to be safe without signs of changes observed in other tissues.<sup>161</sup> A similar approach is to transfect tumor cells with ribozymes that reduce mRNA of VEGF and cause a reduction of more than 70% in the VEGF expression level.<sup>162</sup>

Problems that bedevil gene transfer technology include insufficient distribution of vectors in human tumors and

low transduction efficiency.<sup>163</sup> The development of new strategies targeting tumor angiogenesis is one of the major steps to improve the efficacy of gene therapy, including the role of gene therapy as part of a combined treatment approach.<sup>163</sup>

### *End-points for Determination of Efficacy of Angiosuppressive Agents in Clinical Trials*

The development of antiangiogenesis therapies would be accelerated by the availability of surrogate end-points to determine efficacy of treatment.<sup>164</sup> Attempts to identify angiogenic proteins in the urine or serum,<sup>165</sup> eg, bFGF or VEGF, are problematic and inconsistent. Angiogenesis is a tightly regulated local tissue phenomenon, and systemic metabolic changes such as changes in copper metabolism are not currently well understood. The use of immunohistochemical tissue markers (eg, thrombospondin, CD-31, factor VIII antigen, E-selectin, microvascular density, p53, uPA, uPA receptor) not only may correlate with survival, but also may predict responders and nonresponders to angiosuppressive therapy.<sup>166-173</sup>

No single, reproducible, specific, sensitive serum biomarker exists today, but a major effort remains to develop one. For example, a recent phase I study of SU5416 measured several putative angiogenesis biomarkers, TNF- $\alpha$ , tPA-Ag, IL-8, plasma tissue factor, VEGF, soluble E-selectin, and coagulation tests. There was no change in any of the biomarkers except for a transient decrease in VEGF within 8 hours following the dose.<sup>100</sup>

Noninvasive imaging techniques to monitor antiangiogenesis prospectively are being developed using MR spectroscopy, perfusion MR, vascular permeability changes, or other detectable changes in the microcirculation.<sup>174-176</sup> These techniques may be sufficiently sensitive to detect a decrease in tumor metabolism and mitotic rate while undergoing angiosuppressive therapy.<sup>174</sup> MRI contrast enhancement correlates with microvascular density,<sup>177</sup> supporting our observation that angiogenesis to be a determinant of radiographic contrast enhancement.<sup>178</sup> A standardized yet sensitive method of MRI to detect efficacy of antiangiogenesis therapy would represent a significant advance.

### *Comparison of Clinical Application of Standard Chemotherapy and Angiosuppressive Therapy*

Notable differences between classic cytotoxic chemotherapy and cytostatic antiangiogenesis therapy are summarized in Table 11.

Table 11. — Comparison of Classic Cytotoxic Chemotherapy and Cytostatic Angiosuppressive Therapy		
	Cytotoxic Chemotherapy	Cytostatic Antiangiogenesis
<b>Onset of Effect</b>	Rapid	Slower
<b>End-point for Tumor Response</b>	Reduction in tumor volume	Time to progression/time of survival
<b>Toxicity</b>	Significant (anemia, leukopenia, gastrointestinal, immunological, alopecia)	Mild and well-tolerated; concerns of delayed wound-healing and infertility
<b>Combination Therapy</b>	Multiple agents used frequently. Utilized in conjunction with surgery and radiation therapy.	Synergistic with other antiangiogenic therapies, radiation therapy, cytotoxic chemotherapy, and as adjunct to cytoreductive surgery to prevent recurrence.
<b>Molecular Target</b>	Single or multiple	Single or multiple. Multiple targets (or "panstasis") may be desirable because of "biochemical redundancy" in the control of angiogenesis.
<b>Staging</b>	Selection of chemotherapy regimen determined by organ site	Angiogenesis therapy is dependent on stage and size (ie, preventive for microscopic disease, intervention for small tumor, and

	and the TNM classification	adjuvant for large, end-stage tumor). Organ specificity is not established.

**Less Toxicity:** Because angiosuppressive molecules do not target proliferating epithelial cells, while the endothelium of skin and the gastrointestinal tract are quiescent, angiosuppressive drugs are unlikely to cause bone marrow suppression, gastrointestinal symptoms, or hair loss. However, as with standard chemotherapy or radiation therapy, wound healing could be delayed with antiangiogenesis therapy.<sup>57</sup> Furthermore, angiosuppression could interfere with fertility because of the important role of angiogenesis in ovulation.<sup>179</sup> Angiosuppressive molecules would also be potentially harmful to a fetus because of the role of neovascularization in embryogenesis.<sup>95</sup>

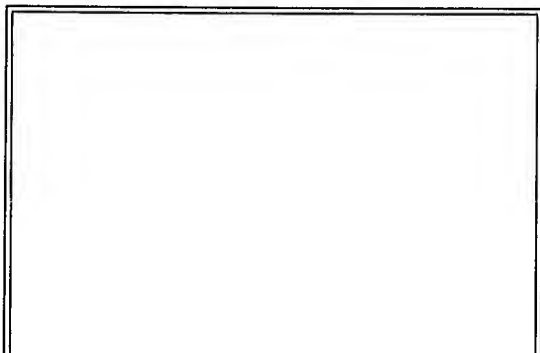
**Tumor Regression vs Disease Stabilization:** Because antiangiogenic drugs do not necessarily kill tumors or dissolve the already established microvasculature, the end-point of early clinical trials may be different than that of standard therapies. Rather than focusing on tumor volumetric response, therapeutic objectives that are more appropriate would be increases in survival time and/or time to disease progression.<sup>6,180</sup> Assessment of efficacy of cytostatic inhibitors needs to avoid the pitfall of requiring tumor shrinkage in order to proceed with clinical development.<sup>180</sup> Classic cytotoxic chemotherapeutic trials seek to define maximal tolerable doses in phase I trials; by contrast, the optimal biological dose is more relevant to trials of therapeutic angiosuppression.<sup>6,180</sup>

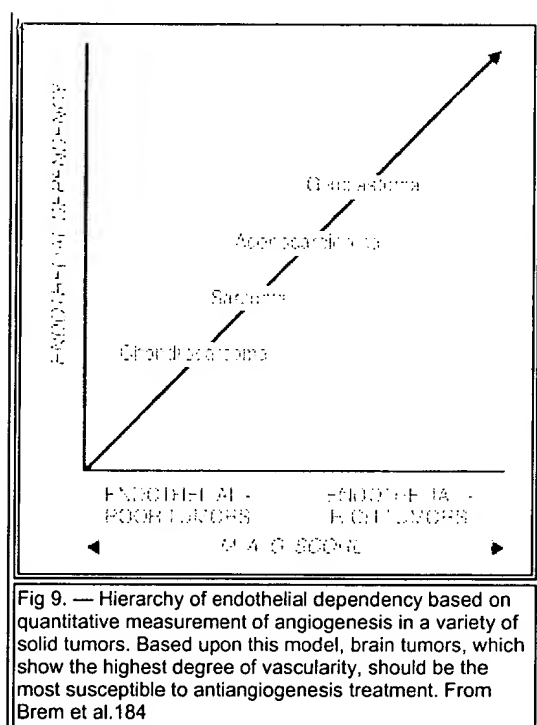
**Drug Resistance:** A major problem with current cytotoxic chemotherapy is drug resistance, in part because the malignant cells are genetically unstable and heterogeneous. By contrast, the target population of antiangiogenic drugs consists of a stable, diploid population of endothelial cells. In long-term preclinical studies, drug resistance to angiosuppressive molecules is not observed.<sup>67,68</sup>

**Combination Therapy:** Although phase I/II studies evaluate individual agents (monotherapy), angiosuppression could prove to be more effective given in combination with standard therapy, eg, chemotherapy,<sup>47,51,94,96,108,150,181,182</sup> radiation,<sup>76-78</sup> surgery, or other antiangiogenic compounds.<sup>22,183</sup>

**Staging of Therapy:** Just as the selection of current cytotoxic chemotherapy is predicated on the specific pathology, anatomic site, and stage of the disease (eg, the TNM classification), it is likely that antiangiogenesis protocols will be site- and stage-specific. In an elegant model of multistage carcinogenesis, Bergers et al<sup>22</sup> recently showed distinct efficacy profiles of four inhibitors of angiogenesis. Three distinct stages of disease progression were defined: the angiogenic switch in premalignant lesions, intervention during the rapid expansion of small tumors, and regression of large end-stage cancers.<sup>22</sup> Newer anticancer therapies are typically introduced for the latter group, but with antiangiogenesis compounds, therapeutic intervention at the earlier stages of disease progression or as a chemopreventive agent may prove fruitful.

As there is currently no drug approved by the Food and Drug Administration for antiangiogenesis, there are many opportunities for drug discovery and development. It is presumed that certain compounds will be more effective for specific types of tumors. We proposed that brain tumors would be the most vulnerable to antagonists of angiogenesis, based on their high degree of microscopic angiogenesis (Fig 9).<sup>184</sup>





## Conclusions

The field of angiogenesis research, once the domain of a few laboratories, has enjoyed spectacular growth since the early work of its pioneers. The NCI has identified angiogenesis research as one of the top cancer research priorities. The 35 biological activators, 18 endogenous inhibitors, and 35 pharmacologic antagonists provide at least 88 reasons for cautious optimism: "It is no longer a question 'if' angiosuppression will work, but rather 'when,' 'what indications,' 'which compound,' 'how much, how fast,' 'what route,' 'what risk,' and 'how long.' Much work remains, but the final chapter of the angiogenesis story should be its most challenging and rewarding."<sup>164</sup>

Several excellent online resources are available to stay current with the rapidly emerging field of angiogenesis inhibitors. The NCI updates information on clinical trials of antiangiogenic therapy on the Angiogenesis Main Page of the NCI's cancer trials Web site at <http://cancertrials.nci.nih.gov>, including a fact sheet on "Angiogenesis Inhibitors in Cancer Research." The information also can be obtained by telephone (Cancer Information Service of the NCI at 1-800-4-CANCER.). The PDQ site can be searched via the Internet or the National Institutes of Health Web search engine at <http://search.info.nih.gov>.

For details of the Penicillamine/Copper Reduction trial or other angiogenesis inhibitors related to brain tumors, recommended Web sites include (1) the NCI's comprehensive cancer data base, PDQ, accessible via CancerNet (<http://cancernet.nci.nih.gov/>), (2) the AI Musella Foundation (<http://www.virtualtrials.com/>), (3) the NABTT home page (<http://www.nabtt.org/>), and (4) the Neurooncology Program at Moffitt Cancer Center's home page (<http://www.moffitt.usf.edu/>).

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